New aspects on the acquisition of biotrophic status by a vesicular–arbuscular mycorrhizal fungus, *Gigaspora margarita*

SUMMARY

An *in vitro* system for culturing the vesicular–arbuscular (VA) mycorrhizal fungus *Gigaspora margarita* Becker & Hall with Ri T-DNA transformed roots of carrot has been used to investigate the progressive dependency of a VA mycorrhizal fungus on its host during mycorrhizal establishment. The growth of hyphae from germinating spores of *G. margarita* was measured under different combinations of the following treatments: in absence or presence of root, with or without mycorrhizal colonization, with or without spore removal. Two distinct mechanisms of hyphal growth stimulation have been identified. The first required the presence of the root but remained spore dependent. The second is exclusively root dependent. It has been demonstrated that the second mechanism is triggered when arbuscules are forming, indicating they are necessary for establishment of biotrophy. It is proposed that roots contribute to fungal growth by an inductive action, in addition to providing nutrients.

Key words: *Gigaspora margarita*, transformed roots, hyphal growth, biotrophy, arbuscule.

INTRODUCTION

Obtaining the growth of vesicular–arbuscular (VA) mycorrhizal fungi in pure culture is an important objective from both the scientific and applied point of view. Taxonomic, physiological and genetic studies of VA mycorrhical fungi, and large scale production of inoculum for field application are considerably limited by our inability to culture and maintain these fungi axenically. These obligate symbionts can be successfully grown and maintained in the presence of a host plant, and they exhibit a short period of independent growth when initiated from germinating propagules (spores or intramatrical vesicles) (Mosse, 1959; Hepper, 1983; Biermann & Linderman, 1983; Strullu & Romand, 1987). Thus the key to success in culturing VA mycorrhizal fungi is to define the contributions of the host plant and fungal propagules (biotic factor) on hyphal growth in the hope of replicating these factors abiotically.

One approach for identifying essential nutrients provided by the host is the quantitative testing of the effect of nutritional factors on the growth of hyphae from germinating spores. Factors such as organic acids, vitamins, amino-acids, sulfur-containing compounds, protein hydrolysates and plant extracts improved, to some extent, hyphal growth but they were insufficient to maintain growth when hyphae were separated from the spores and subcultured (Hepper, 1984). Such observations raise questions about the intimate mechanisms associated with the presence of spores and also reinforce the view that critical factors are still to be discovered for the growth of VA mycorrhizal fungi.

Axenic culture of some species of VA mycorrhizal fungi has been successfully achieved using root organ cultures (Mosse & Hepper, 1975; Miller-Wideman & Watrud, 1984; Mugnier & Mosse, 1987). Dual culture is initiated using fungal inocula such as spores or mycorrhizal root segments. This type of approach allows nondestructive observations of fungal development and can be used to increase our knowledge of plant contributions to hyphal growth. However, these techniques are not sufficiently simple and reproducible to allow standardized quantitative measurements of fungal growth. Bécard and Fortin (1988) recently developed a simple and reproducible system to study VA mycorrhizal development based on infection of carrot roots which have been transformed by the T-DNA of the Ri plasmid of *Agrobacterium rhizogenes*.

Using this defined system, we measured the influence of spore reserves, root stimulation, and

root colonization on hyphal growth. We also present a model for interpreting the relationship between spore and root, and their role during the establishment of the VA mycorrhizal symbiosis.

MATERIALS AND METHODS

Root organ culture

A clone of Ri T-DNA transformed root of carrot (*Daucus carota* L.) was established and cultured as previously described by Bécard & Fortin (1988). For routine maintenance, the roots were grown on a modified White's (MW) medium and for the growth and development of dual culture, a minimal (M) medium was used (Bécard & Fortin, 1988).

Fungal inoculum

Spores of *Gigaspora margarita* Becker & Hall (DAOM 194757, deposited at the Biosystematic Research Center, Ottawa, Canada) were recovered from leek (*Allium porrum* L. cv. Titan) pot cultures by wet sieving (Gerdemann & Nicolson, 1963) and subsequently purified by density gradient centrifugation (Furlan, Bartshi & Fortin, 1980). Isolated spores were surface sterilized, stored at 4 °C and resterilized before use according to the procedure of Bécard & Fortin (1988).

Dual culture system

Dual culture was achieved by placing a single spore with a single transformed root of carrot in a square (9 × 9 cm) Petri dish to constitute what is considered as an experimental unit. A non-germinated spore of *G. margarita* was positioned in the middle of each dish and pushed gently into the M medium. The Petri plates were then placed in a vertical position at 27 °C in order to permit the upward growth of each emerging germ tube. Two sterile cotton rolls (dental rolls Healthco, DDL Montreal Canada) were placed in the bottom of each Petri plate to absorb the excess water. Three days after spore germination, the dual culture was initiated by placing selected roots horizontally 5–6 mm above the growing germ tubes, so that intimate contact occurred within the next day between the germ tube and the elongation zones of the root (4–5 cm behind the apex). These root explants which were introduced near the germ tubes were produced as follow: 10 mm root apices were cut from the routine culture (MW) and then grown on M medium in inverted Petri plates at 27 °C for 21 d. This time period allows root apices to grow as much as 20 cm long with many lateral roots and have vigorous elongation zone. These 21-d-old main roots were cut again 10 cm behind the growing apex and transferred into dishes containing germinated spores.

Table 1. *Experimental treatments*

With a dialysis membrane (without root colonization)	Absence of dialysis membrane (with root colonization)
With or without the presence of roots Spores removed after 5 d of dual culture*	Spores removed after 8 d of dual culture* Spores removed after 5 d of dual culture*
Roots removed after 5 d of dual culture*	
Spores removed after 8 d of dual culture*	

* Spores or roots were removed from the dual culture using a sharp pointed scalpel or a pair of tweezers. The spore extraction was made easier by using a red-hot scalpel on which the spores adhered after cutting of the germ tube.

Experimental treatments

Two different types of experiments were performed using the dual culture system:

(*a*) Use of a dialysis membrane between the symbiotic partners in order to avoid hyphal contact with roots and thereby root colonization.

(*b*) Absence of dialysis membrane which allowed contact and root infection by *G. margarita* germ tubes.

In both types of experiments, the effect of several different treatments were studied (Table 1). These treatments were chosen in order to understand the relative contributions of spore/root to germ tubes and hyphal growth of *G. margarita*.

The dialysis membrane had a molecular weight cut-off of 12000–14000 (spectra/por 2, Spectrum medical industries, Inc.). They were washed for at least 24 h in distilled water and autoclaved at 121 °C for 15 min. They were then laid on to the agar media and overlaid with a thin layer of M medium. The spores were inserted underneath the membrane through a small incision. The roots were placed over the membranes which covered the upper half of the Petri dishes completely [see Fig. 1, (no. 1)] such that hyphal contact with the roots was not possible.

Assessment of extraradical and intraradical fungal growth

The elongation of the extraradical hyphae was recorded daily under a binocular lens for each experimental unit. The linear growth of hyphae was marked on the back of the Petri plates and measured by counting the number of crossed squares using a grid of 2 mm squares [see Fig. 1, (nos. 1,2)]. The treatments previously described, were applied at least twice on 6–10 experimental units. The various effects on hyphal elongation were interpreted in terms of extraradical growth curve patterns.

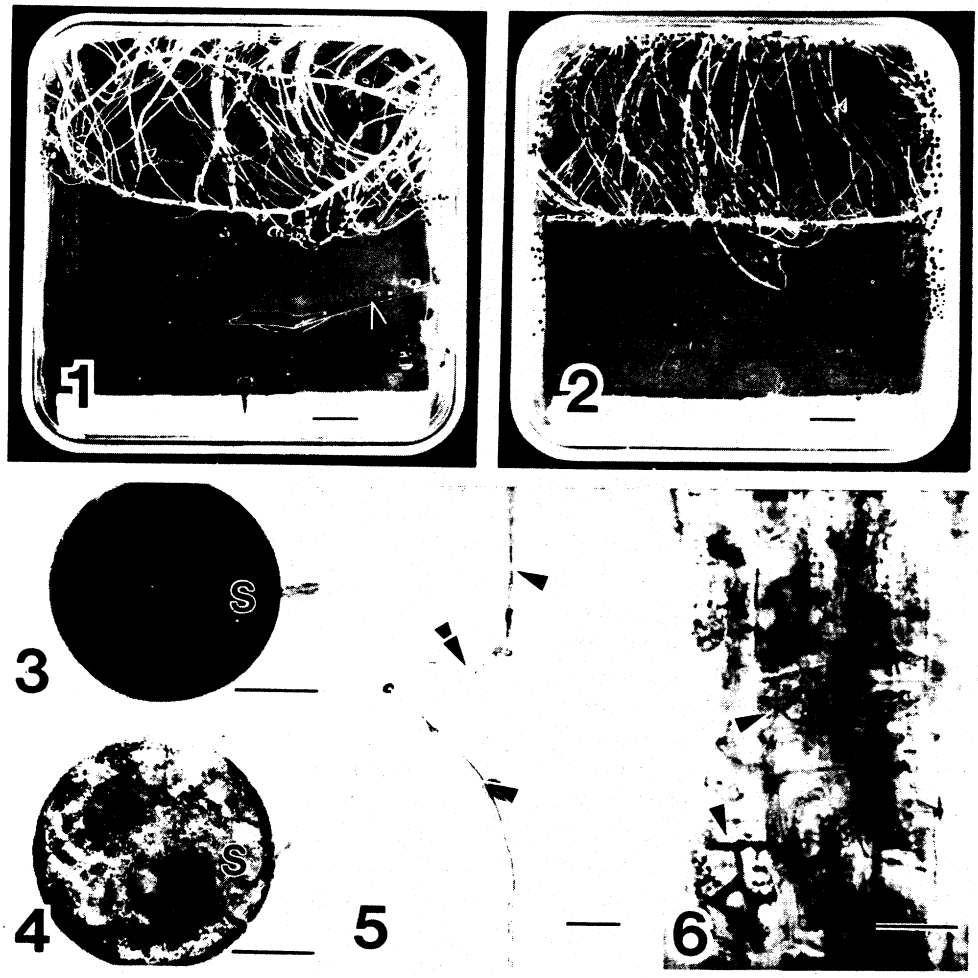

Figure 1

Nos. 1,2. Dual culture of a Ri T-DNA transformed root of carrot with a germinating spore of *Gigaspora margarita*. The dots were made to measure hyphal elongation. Bar equals 1 cm.

No. 1. Completed hyphal growth (400 mm) from one germinating spore of *Gigaspora margarita* after 3 weeks of dual culture without root colonization (presence of a dialysis membrane, arrow).

No. 2. Hyphal growth in progress from one germinating spore of *Gigaspora margarita* after 5 weeks of dual culture with root colonization. The spore was removed after 8 d of dual culture.

Nos. 3,4. Low magnification of 2 *Gigaspora margarita* spores (S) 4 weeks after germination using the same transmitted light. Bar equals 100 μm.

No. 3. In absence of root, the content of the spore looks dense and opaque.

No. 4. In presence of root without root colonization, the spore looks almost emptied.

No. 5. Hyphal healing (double arrow) between the two divided sections of a germ tube (simple arrow) of *Gigaspora margarita* when the spore is left *in situ*. Bar equals 100 μm.

No. 6. Arbuscules of *Gigaspora margarita* (arrow) in Ri T-DNA transformed roots of carrot. Bar equals 30 μm.

When contact between hyphae and roots was permitted, intraradical hyphal growth was also assessed. Prior to these observations, root segments were cleared in 10 % KOH (w/v) for 10 min, rinsed in water and stained in 0·1 % Chlorazol Black E (w/v) for 2 h (Brundrett, Piché & Peterson, 1984). Three sequential stages in the development of the infection were distinguished after root staining:

- attachment of the fungus to the root surface without any hyphal penetration of the root (appressoria);
- hyphal penetration of the root following attachment (appressoria + penetrations);
- intracellular spread with arbuscules formation, following fungal attachment and root penetration (appressoria + penetrations + arbuscules).

These stages were observed under a microscope at the same magnification and in the same field of view but at three successive focal planes.

RESULTS

First mechanism of fungal growth promotion

In the absence of roots, hyphal elongation was slow and reached a maximum about 10 d after germination (Fig. 2,a). Germ tubes either produced some branches and auxiliary cells, or they extended only 20–30 mm without branches. There was a significant increase (20-fold) in hyphal growth when roots were introduced 3 d after spore germination without establishment of physical contact i.e. without root colonization (Fig. 2,b). Under these conditions, the hyphae demonstrated logistic growth with a rapid increase in growth rate at the start (3–6 d) and then decreasing progressively to zero (15–28 d). No apparent correlation between length of elongated hyphae and spores size was found. Fungal growth was completed 4 weeks after spore germination [Fig. 1 (no. 1); Fig. 2,b] with an average of 400 mm of hyphae extending from the spores. At this stage, internal content of the spores looked less dense and organized compared to spores with elongated hyphae that were grown in absence of roots [Fig. 1 (nos. 3,4)]. When the spore was severed from the germ tube and removed after five days of dual culture, hyphal extension gradually stopped within the next 2 d (Fig. 3,a). Under no circumstances was the severed spore left in the culture dish. Indeed, the fungus was capable of forming a junction between its spore and germ tube, thus restoring elongation at the hyphal tips [Fig. 1 (no. 5)]. A rapid decrease in fungal growth was observed when the root was removed (Fig. 3,b). These results reveal the presence of a first mechanism (M1) which stimulates fungal growth and which requires the continuous presence of both the root and the spore.

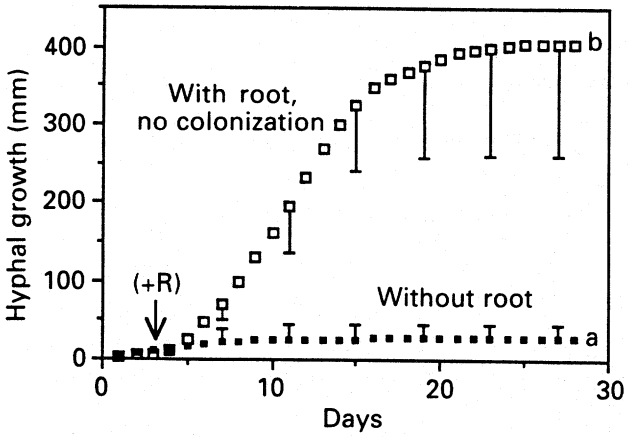

Figure 2. Hyphal growth (mm) from germinating spores of *Gigaspora margarita*. Symbols: ■, curve a, in absence of root; □, curve b, in presence of root (+R) and without root colonization. Vertical lines correspond to the standard deviation. Each point is a mean of ten replicates.

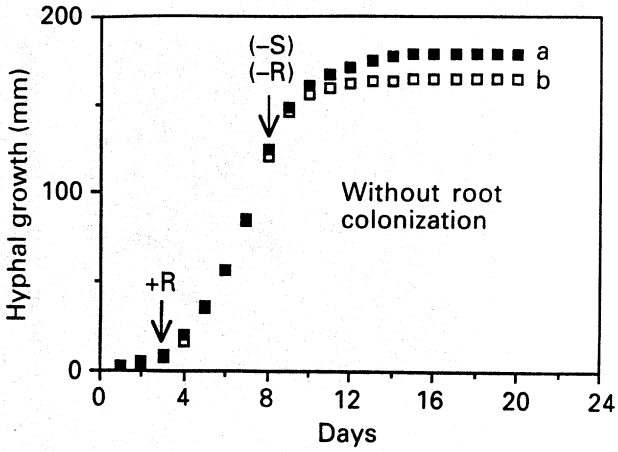

Figure 3. Hyphal growth (mm) from germinating spores of *Gigaspora margarita* in presence of root (+R) but without root colonization. Symbols: ■, curve a, the spore (−S); or □, curve b, the root (−R) were removed after 5 d of dual culture. Each point is a mean of six replicates.

Second mechanism of fungal growth promotion

After a period of 8 d of dual culture, when germ tubes were permitted to make contact with the roots, complete colonization (100%) of the roots was observed (Table 2). In addition, extramatrical hyphal elongation persisted even after the removal of the spore (Fig. 4,a). A 5-week-old dual culture under these conditions is shown where at least one infection unit had occurred [Fig. 1 (no. 2)]. The hyphae had spread all over the Petri plate and under these circumstances, spore production can be obtained if dual culture is maintained for a long enough term (Bécard & Fortin, 1988). As in a preceding experiment (Fig. 3,a), hyphal growth stopped rapidly two days after spore removal when root colonization was prevented (Fig. 4,b). This constitutes a second mechanism (M2) by which the root promotes fungal growth without the requirement of the spore.

The critical intramatrical phase in M2 fungal growth promotion

After a period of 5 d of dual culture, when contact between germ tubes and roots was permitted, the colonization process was in progress (Table 2). Only

Table 2. *Colonization process after 5 and 8 d of dual culture. Values represent percentage of 10–15 main roots*

Days of dual culture	Appressoria (%)	Appressoria + penetrations (%)	Appressoria + penetrations + arbuscules (%)
5	21	21	7
8	0	0	100

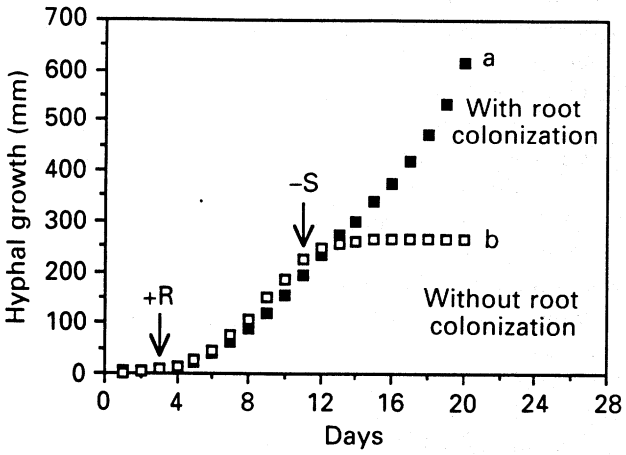

Figure 4. Hyphal growth (mm) from germinating spores of *Gigaspora margarita* in presence of root (+R). Symbols: ■, curve a, with root colonization; or □, curve b, without root colonization both having the spore removed (−S) after 8 d of dual culture. Each point is a mean of six replicates.

7 % of the root had completed intraradical colonization up to the stage of arbuscule formation. When the spore was removed at this stage in a second set of experimental units, three different curves of extraradical growth were obtained (Fig. 5). In 60 % of the cases, extraradical growth ceased and the roots showed either incomplete or no infection. Only in 7 % of the cases did the hyphae continue to grow; while in 33 % of the cases hyphae showed growth revival after a period of cessation. Except for the latter case where roots were immediately examined on the day when growth revived, the roots were examined 10 d after spore removal. In the latter two cases the roots were completely colonized with arbuscules [Fig. 1 (no. 6)]. When there was a

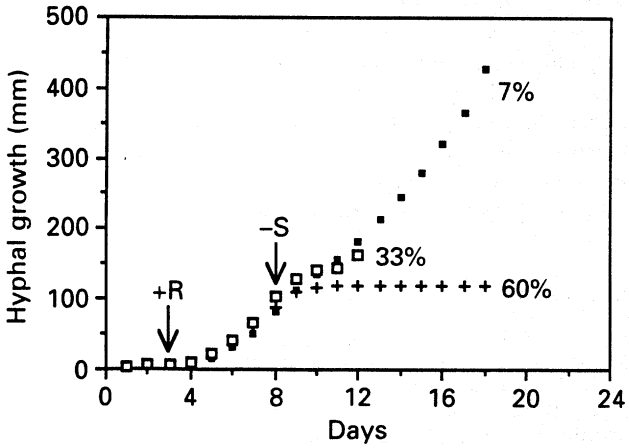

Figure 5. Hyphal growth (mm) from germinating spores of *Gigaspora margarita* in presence of root (+R). Three kinds of growth curve patterns were obtained, depending of the root colonization progress, when the spore (−S) were removed after 5 d of dual culture. Percentages represent the obtained proportions, based on 15 experimental units, of the corresponding growth curve patterns.

temporary pause in fungal growth after spore removal, the pause was probably required, during the 2 d of growth inertia, for the development of arbuscules which then allowed the growth independent of spores of the extraradical phase. These results demonstrate the involvement of arbuscules in the triggering of the sporal independent growth of the extraradical phase, i.e. in the triggering of the second mechanism (M2). They also demonstrate that the formation of the intraradical phase up to arbuscule formation is spore dependent.

DISCUSSION

The first experiment (Fig. 2,a) illustrated the inability of VA mycorrhizal fungi to grow in pure culture despite the large quantities of energy stored in their spores in the form of lipids (Jabaji-Hare, 1988). Understanding why a germinating spore is incapable of catabolizing its own reserves is important before we can attempt to culture VA mycorrhizal fungi. A partial explanation may have been given by Watrud, Heithaus & Jaworski (1978) who reported an improvement of hyphal growth from spores of *G. margarita* in the presence of 0·01 % activated charcoal, thus suggesting a form of autoinhibition in this fungus.

Under our conditions, the presence of a growing root stimulated significantly the growth of the fungus even when there was no root-fungal contact. This result has also been found for *Glomus mosseae* (Mosse & Hepper, 1975) in root organ culture. It can also be related to the work of Mosse (1959) who showed that sonicated roots stimulated hyphal growth, and to that of Carr *et al.* (1985) who demonstrated a similar effect with undifferentiated plant cells. The three following observations indicated that the presence of a root enabled the fungus to utilize its reserves as the sole source of food (mechanism M1): the structural alteration of the spore during hyphal growth which was probably due to the consumption of reserves [Fig. 1 (nos. 3, 4)], the progressive cessation of hyphal growth 3 weeks after spore germination (Fig. 2,b) and the rapid cessation of hyphal growth when the spore was removed (Figs 3,a; 4,b). The root may have exerted its influence by changing the composition of the agar medium or the gaseous phase in the dual culture system. Root exudates have been reported to promote hyphal growth from spores (Mosse & Hepper, 1975). However, since active fungal growth was rapidly lost after root removal (Fig. 3,b) it would appear that either the active components in root exudates were not stable or they did not accumulate in sufficient quantities.

The characterization of M1 is important if we are to replace roots and spores by abiotic factors in the cultivation of VA mycorrhizal fungi, but it does not appear sufficient since fungal growth still requires the presence of the spore.

A re-evaluation seems necessary of the numerous works which have related the effects of different chemical factors on the growth of hyphae from germinating spores. Although root exudates, plant extracts or different carbon and nitrogen sources have occasionally improved the growth of hyphae (Gerdemann, 1955; Mosse, 1959; Hepper, 1979; 1983), all the attempts to subculture the stimulated hyphae have failed, even when all known stimulatory factors were incorporated in the medium. It would appear impossible to obtain hyphal growth without spores. It is our opinion that the tested nutritional factors are not necessary for fungal growth when spore reserves are still available. Moreover, the utilization of spores reserves requires specific environmental conditions which may also be required for the utilization of other nutritional sources in the absence of spores. These conditions apparently promote an activated form for the fungus and they are apparently provided by roots through the first mechanism M1. This root induction may be a chemical signal that directly stimulates fungal growth, or a removal of fungal self-inhibition.

This study demonstrates for the first time the essential role of arbuscules in the supply of food by the roots. Both appressoria and intraradical hyphae were not able to replace the spore as the food supply for external hyphae. This is apparently in contrast to the observations of Mosse & Hepper (1975) and of Hepper (1981) who reported a stimulation in growth of external hyphae during appressoria formation or during penetration of hyphae between the root cells. However, these authors had not used the criterion of spore dependence for hyphal growth to define a new phase of fungal growth. Our results increase the interest for closer examination of the chemicals translocated from plant to fungal symbiont at the interface between arbuscules and host cells. These exchanges are primary components of a second mechanism (M2) by which the root contributes to fungal growth. Although the nutritional source for the fungus has changed, M2 may be similar to M1 in that the same root induction is required for fungal growth.

From the ecological point of view, it is interesting that spores of *G. margarita* have good germination potential but do not consume all their reserves when roots are not in close proximity. Since aborted germinations produce approximately 20 mm of hyphae (Fig. 1, a) and since complete germinations can produce 400 mm of hyphae (Fig. 2, b), a *G. margarita* spore is theoretically capable of 20 germinations. This capability for multiple germinations has been assessed experimentally by Koske (1981) with *Gigaspora gigantea*. When germ tubes were periodically severed, the spores were able to germinate up to ten times. When roots are not in close proximity, fungal growth stops quite rapidly and can restart as soon as roots are introduced (data not shown). The revival of growth may involve either rejuvenation of hyphal growth or regermination of the spore. This tendency of *G. margarita* spores to protect their reserves permit them to remain infective after a series of perturbations and thus they have the potential to infect different plants at different times. It is possible that the resting spores extracted from soil or pot culture have already undergone a series of germinations. This may explain why they show a variability in germination delay and hyphal growth potential and they show no correlation between spore size and hyphal growth.

From our results, we propose a model for the growth of VA mycorrhizal fungi from the germinating spore to the achievement of the symbiotic state. Hyphal growth from a germinating spore initially depends on nutritional reserves in the spore and is slow and limited. The presence of roots induces the fungus, by direct activation or by removal of inhibitors, to enter a second phase of growth which is characterized by stimulation of hyphal growth. This second phase of growth is still dependent on and limited by spore reserves. This is the first mechanism of root contribution to fungal growth (M1). The fungus takes advantage of M1 to spread, to achieve contact with roots and to subsequently infect the roots. A third phase of fungal growth begins when a second mechanism (M2) is initiated, where the development of arbuscules enables the fungus to utilize the root as a nutritional source. The root's contribution to fungal growth therefore arises from at least two factors which probably act conjointly during the symbiotic state, one is inductive and the other is nutritional.

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